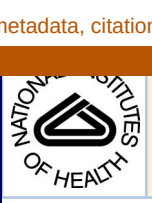

Published in final edited form as:

Circ Res. 2015 January 16; 116(2): 237–244. doi:10.1161/CIRCRESAHA.116.305547.

Stoichiometry of Gata4, Mef2c, and Tbx5 Influences the Efficiency and Quality of Induced Cardiac Myocyte Reprogramming

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Abstract

Rationale—Generation of induced cardiac myocytes (iCMs) directly from fibroblasts offers great opportunities for cardiac disease modeling and cardiac regeneration. A major challenge of iCM generation is the low conversion rate of fibroblasts to fully reprogrammed iCMs, which could in part be attributed to unbalanced expression of reprogramming factors Gata4 (G), Mef2c (M), and Tbx5 (T) using the current gene delivery approach.

Objective—We aimed to establish a system to express distinct ratios of G, M, T proteins in fibroblasts and determine the effect of G, M, T stoichiometry on iCM reprogramming.

Methods and Results—We took advantage of the inherent feature of the polycistronic system and generated all possible combinations of G, M, T with identical 2A sequences in a single transgene. We demonstrated that each splicing order of G, M, T gave rise to distinct G, M, T protein expression levels. Combinations that resulted in higher protein level of Mef2c with lower levels of Gata4 and Tbx5 significantly enhanced reprogramming efficiency compared to separate G, M, T transduction. Importantly, after further optimization, the MGT vector resulted in more than 10-fold increase in the number of mature beating iCMs loci. Molecular characterization revealed that more optimal G, M, T stoichiometry correlated with higher expression of mature cardiac myocyte markers.

Conclusion—Our results demonstrate that stoichiometry of G, M, T protein expression influences the efficiency and quality of iCM reprogramming. The established optimal G, M, T expression condition will provide a valuable platform for future iCM studies.

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DISCLOSURES

None.

Keywords

iCM; cardiac myocyte; fibroblasts; cell reprogramming; Gata4; Mef2c; Tbx5; transcription factors; gene expression/regulation; regeneration

INTRODUCTION

The generation of iCM from fibroblast holds great promise for cardiac disease modeling and regenerative medicine[1-15]. However, the inefficient iCM generation has become a major hurdle for deciphering the mechanism of cardiac reprogramming and *in vitro* cardiac disease modeling[7, 16]. Although the low conversion rate of fibroblasts to reprogrammed iCMs suggests the existence of major rate limiting barrier(s), it might also reflect a requirement for a balanced expression of Gata4(G), Mef2c(M), Tbx5(T) to promote successful and complete reprogramming. Current iCM generation involves transducing fibroblasts with pooled viruses encoding the three individual reprogramming factors. This approach suffers from heterogeneous and uncontrollable ratios of G, M, T expression among the transduced fibroblasts. Inagawa et al. attempted to address this issue through the use of a polycistronic vector that encoded G, M, T in a single transgene; however, their study showed only a marginal positive effect on reprogramming efficiency when compared to use of individual viruses[9].

Gata4, Mef2c and Tbx5 are the master regulators residing at the top of the transcriptional hierarchy of the cardiac gene regulatory networks[17-33]. During heart development, faithful execution of cardiac developmental processes requires a precise dosage and temporal expression of these three factors[17-33]. Disruption of this delicate balance is likely to compromise cardiac specification and differentiation, and cause severe cardiac anomalies[24-26, 34-36]. We therefore postulated that when G, M, T were delivered in separate viruses, only a subpopulation of CFs would express the optimal amounts of G, M, T required to trigger cardiac reprogramming. Thus we surmised that it might be possible to establish a more appropriate balance of G, M, T expression in non-myocytes through the use of splice-ordered polycistronic vectors.

In this study, we generated a complete set of polycistronic constructs containing G, M, T in all possible splicing orders with identical 2A sequences in a single mRNA. We found that each splicing order of G, M, T gave rise to distinct ratios of G, M, T protein expression, and significantly different reprogramming efficiencies. Upon further optimization, the most desirable combination resulted in a more than 10-fold increase in generation of beating iCMs. Importantly, at the molecular level, the more optimal G, M, T stoichiometry, defined by higher protein expression level of Mef2c with lower levels of Gata4 and Tbx5, correlated with higher expression of mature cardiac myocyte markers. Thus, our study demonstrates that stoichiometry of G, M, T influences both efficiency and quality of iCM induction.

METHODS

An expanded Methods section is available in the online data.

RESULTS

G, M and T protein levels differ in fibroblasts expressing each of the 6 polycistronic constructs

To manipulate the relative levels of G, M, T protein expression, we generated 6 polycistronic constructs with identical 2A sequences to include all possible splicing orders of G, M, T in a single mRNA (Fig. 1A). In a first set of experiments, we transduced cardiac fibroblasts (CFs) with G, M, or T retroviruses separately. Western blot analysis showed that G, M, or T proteins were detected at the appropriate molecular weight (Online Fig. IA). Next we performed western blot on CFs transduced with each of the 6 constructs and observed higher protein expression level when the reprogramming factor was placed at the 5' end vs. 3' end of the construct (Fig. 1B,C). After normalization to the loading control, quantification of band intensities revealed that each construct produced distinct ratios of G, M, T protein expression (Fig. 1B,C and Online Fig. IB). We also determined G, M, T transcript levels in transduced cells (Online Fig. II). As expected, after virus infection, G, M, T transcript levels were significantly elevated, however the CFs expressing each of these 6 constructs did not exhibit significant differences in G, M, T total and endogenous transcripts levels (Online Fig. II). These data suggest that our complete set of polycistronic vectors can effectively produce distinct ratios of G, M, T protein expression in transduced CFs.

Different iCM reprogramming efficiency using each of the 6 polycistronic constructs

We next sought to determine iCM reprogramming efficiency using these 6 polycistronic constructs. We isolated CFs from αMHC-GFP reporter mice[1, 2], which express GFP in differentiated cardiac myocytes (CMs) but not in CFs. Thus, activation of GFP could allow us to follow the emergence of newly induced iCMs. Furthermore, we used cardiac TroponinT (cTnT) as an additional differentiated CM marker to monitor CM fate induction. We transduced CFs with retroviruses encoding the 6 polycistronic mRNAs. Interestingly, flow analysis and quantification showed that these 6 vectors resulted in significantly different reprogramming efficiencies as indicated by the differences in the percentage of αMHC-GFP+ and cTnT+ cells (Fig. 2A,B). Noticeably, only 2 vectors (MGT and MTG) enhanced reprogramming efficiency compared to separate G, M, T vectors while the other 4 resulted in a decrease in reprogramming efficiency (Fig. 2A,B). Similar results were obtained by performing immunocytochemistry (ICC) with antibody against αMHC-GFP (Fig. 2C and Online Fig. III). Likewise, western blot analysis of CFs reprogrammed with the 6 different vectors indicated that reprogramming with MGT resulted in the highest up-regulation of αMHC-GFP and α-actinin protein expression (Fig. 2D). Based on the two analyzed markers, these data suggest that the 6 polycistronic constructs conferred differential effects on iCM induction. While the experiments were performed with neonatal CFs, these polycistronic vectors exhibited similar relative reprogramming efficiencies (based on the percentage of αMHC-GFP+ iCMs) in neonatal tail tip fibroblasts (TTFs), adult CFs and adult TTFs (Online Fig. IV). We then focused on the vector with the highest efficiency (MGT) and performed additional experiments to determine and quantify its reprogramming efficiency relative to that of separate G, M, T vectors (Online Fig. V, Online Fig. VI). Collectively, our results demonstrate that the 6 polycistronic vectors exhibit significantly different iCM reprogramming capacities, and a more optimal G, M, T

stoichiometry, defined by higher protein level of Mef2c with lower levels of Gata4 and Tbx5, significantly increases iCM reprogramming efficiency.

Enhancement of the quality of iCM reprogramming using MGT with antibiotic selection

To determine if a more optimal G, M, T stoichiometry of the MGT vector could be utilized to improve functional maturation of iCMs, we added a puromycin resistance gene in the vector (puro-MGT) to enable selection of transduced CFs (Fig. 3). By utilizing puro-MGT to reprogram freshly isolated CFs, we observed a 3-fold increase in the percentage of αMHC-GFP+ cells and 5-fold increase in the percentage of cTnT+ cells (Fig. 3A,B). To obtain a global view of iCMs, we took 228 snapshots of 10X ICC pictures to cover a full well of a 24-well plate. After stitching all pictures together, we observed a population of cells that were highly enriched with iCMs, which frequently aggregated to form clusters (Fig. 3C). These iCMs formed sarcomere structures (stained for cTnT and α-actinin) resembling the ones of fetal cardiac myocytes and expressed the gap junction protein Connexin43 (Cx43) at the cell-cell contacts (Fig. 3D). We next analyzed intracellular Ca^{2+} flux by Rhod3 dye labeling after 4 weeks of culture. With puromycin selection, around 35% of transduced cells showed spontaneous Ca^{2+} oscillations and their frequency was variable, as previously reported in reprogrammed iCMs[11]. Figure 3E shows a representative iCM-rich area containing 8 iCMs. Intracellular Ca^{2+} concentration in this area was recorded in 4 loci, and 3 of them showed periodic Ca^{2+} transients (#1-3 representative traces) suggestive of advanced reprogramming stages, while the fourth trace showed aperiodic oscillations. The number of Ca^{2+} transient-expressing loci was increased by application of puromycin selection (Fig. 3E). In addition to periodic Ca^{2+} transients, MGT-transduced iCMs showed spontaneous contractile activity after 3 weeks in culture (Online Movies I and II). With increase in culture time to 6 weeks, the number of beating iCM loci generated using the puro-MGT vector increased to 30-40 loci per well of a 24-well plate (Fig. 3F), which was over ten-fold higher than previously reported numbers of beating loci generated by using the same or similar reprogramming factors[1, 8]. Collectively, our data suggest that a more optimal stoichiometry of the reprogramming factors affords a more efficient and complete conversion of a CF into a CM fate.

Molecular characterization of reprogramming cells resulted from varied G, M, T stoichiometry

Next we sought to determine how G, M, T protein stoichiometry influenced iCM gene expression profile. We performed real time quantitative PCR (qPCR) using a set of CM and CF markers that were previously used to characterize iCMs[3] (Fig. 4). Interestingly, the 6 constructs led to significant differences in CM marker gene expression. The most efficient MGT and MTG vectors resulted in a higher upregulation of sarcomere structure genes Myh6, Myl7, Tnnt2, Actc1, muscle contractility genes Pln, Slc8a1, Scn5a, gap junction protein gene Gja1 and ion channel genes Kcnj2, Cacba1c (Fig.4). Noticeably, we observed a reverse correlation of upregulation of cardiac stress genes Nppa and Nppb expression with reprogramming efficiency (Fig.4 D,E). The four constructs that resulted in a decreased reprogramming efficiency upregulated Nppa and Nppb genes expression (Fig. 4D,E), possibly due to over-activation by high levels of Tbx5 and Gata4. In contrast, fibroblast markers such as Col1a1, Col3a1, Eln were all significantly downregulated with no

significant difference among the 6 constructs, suggesting that they exhibited a similar capacity to repress fibroblast markers gene expression (Fig. 4L-N). Taken together, these data suggest that the stoichiometry of G, M, T influences not only the efficiency of iCM induction, but also the activation of cardiac myocyte markers in iCMs.

DISCUSSION

In this study, we took advantage of the inherent feature of polycistronic vectors to manipulate the protein expression levels of cardiac reprogramming factors G, M, T, and reveal a critical role of G, M, T protein stoichiometry in iCM reprogramming. We established that the optimal G, M, and T expression for reprogramming is a relative high level of Mef2c expression and low levels of Gata4 and Tbx5 expression. In addition, our studies suggest that an optimal balance among these three reprogramming factors could allow more efficient and complete conversion of CFs into cardiac myocyte-like cells.

To the best of our knowledge, this is the first work that has built a complete set of polycistronic vectors for a reprogramming cocktail. Carey et al. has previously compared the iPSC reprogramming between two existing polycistronic transgenes (OSKM v.s. OKSM)[37]. They demonstrated that these two transgenes conferred differential effects on iPSC induction, possibly due to the differences in O (Oct4), S(Sox2), K(Klf4), M(cMyc) protein expression levels as a result of the splicing order of O, S, K, M[37]. However, the two transgenes were generated by different research groups in a way that the backbone of the vectors and the polycistronic cleavage sites were not identical. In this study, we generated a whole series of polycistronic constructs to include all possible splicing orders of cardiac reprogramming factors in the same construct with identical 2A peptides. Our studies demonstrated that difference in protein stoichiometry of G, M, T alone is sufficient to confer a significantly different effect on cardiac reprogramming outcomes. It will be important to determine if the inter-regulatory relationships among the three factors play a role in controlling and maintaining the optimal balance of G, M, T expression needed for reprogramming. In addition to the polycistronic system, independent approaches such as those utilizing promoters with different strengths to manipulate the relative levels of G, M, T protein expression could be undertaken to further determine the effect of stoichiometry on iCM reprogramming. It remains to be determined if optimized stoichiometry of human G, M, T could be sufficient to induce cardiac fate in human fibroblasts without the use of additional factors and/or microRNAs[13-15]. Moreover, we found that the unbalanced protein expression of G, M, T (when using TMG, TGM, GMT, and GTM vectors) led to an inefficient iCM reprogramming. While these unbalanced G, M, T combinations were equally capable of suppressing gene expression of fibroblast markers, they failed to robustly activate cardiac program; and instead induced cardiac stress genes Nppa and Nppb. Our results thus could explain some discrepancies among different groups that attempted G, M, T-mediated iCM induction [7, 9, 16]. For example, similar to G, M, T expression when using our TGM construct, the inefficient cardiac reprogramming reported by Chen et al[7] was likely caused by relatively high expression of Tbx5 and Gata4 as well as the enrichment for potential iCMs based on expression of Tbx5. The marginal effects of using a polycistronic construct TMG reported by Inagawa et al[9] could be similarly attributed to the non-optimal stoichiometry of reprogramming factors.

In spite of excitement and significant potential of iCM reprogramming in regenerative medicine, the low conversion rate of fibroblasts into iCMs has been a major challenge for future translational efforts. Although this low efficiency is expected, as it has been reported for other reprogramming technologies during their fledgling phase[38], there are ongoing efforts to overcome the hurdles and to identify small molecules to replace transcription factors for therapeutic purposes. All of these attempts would not be fruitful without a consistent and reproducible platform. Our most efficient polycistronic construct that has been further modified by adding antibiotic selection (pMX-puro-MGT) not only eliminates the need for multiple constructs, providing a homogenous gene expression stoichiometry, but also allows for further enrichment of transduced cells with antibiotic selection. This platform offers a reasonable starting point for future screening and/or mechanistic studies, especially those that require large amounts of materials to explore genome-wide molecular changes that occur during reprogramming. In addition, our complete set of polycistronic constructs can be further used to study the optimal strategies to convert other non-myocytes in addition to fibroblasts into cardiac myocytes, offering insights into how stoichiometry of G, M, T expression can control cardiac fate acquisition under a variety of conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful for the expert technical assistance from the UNC Flow Cytometry Core and UNC Microscopy Core. We thank Dr. Joan Taylor and members of the Qian lab and the Liu lab for helpful discussions and critical reviews of the manuscript.

SOURCE OF FUNDING

This study was supported by NIH/NHLBI 1R01HL104326 grant to Dr.Bursac, NIH/NHLBI R00 HL109079 grant to Dr. Liu and American Heart Association (AHA) Scientist Development Grant 13SDG17060010 and the Ellison Medical Foundation (EMF) New Scholar Grant AG-NS-1064-13 to Dr. Qian.

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Nonstandard Abbreviation and Acronyms

αMHC/Myh6	alpha muscle heavy chain, also known as myosin heavy chain 6
CF	cardiac fibroblast
CM	cardiac myocyte
G, M, T	Gata4, Mef2c, Tbx5
cTnT	cardiac Troponin T
iCM	induced cardiac myocyte
iPSC	induced pluripotent stem cell
TTF	tail tip fibroblast
O, S, K, M	Oct4, Sox2, Klf4, cMyc

Novelty and Significance

What Is Known?

- Fibroblasts can be directly reprogrammed into cardiac myocyte–like cells (called iCMs).
- A combination of Gata4 (G), Mef2c (M), and Tbx5 (T) is sufficient to convert fibroblasts into iCMs.
- Generation of iCM *in vivo* has been shown to improve heart function and reduce scar size in a mouse myocardial infarction model.

What New Information Does This Article Contribute?

- Stoichiometry of G, M, and T protein expression plays important roles in iCM reprogramming.
- The desirable G, M, and T expression for reprogramming is a relative high level of Mef2c protein expression and low levels of Gata4 and Tbx5 expression.
- An optimal balance of G, M, T could allow more efficient and complete conversion of fibroblasts into iCMs.

Direct conversion of fibroblasts into cardiac myocyte-like cells (iCMs) using defined factors such as Gata4 (G), Mef2c(M) and Tbx5(T) holds great promise for regenerative medicine. However, the low conversion rate and the considerable variability in iCM generation have hindered further mechanistic studies and optimization for clinical applications. We took advantage of the inherent feature of the polycistronic system and generated a complete set of polycistronic constructs to include all possible splicing orders of G, M, T in a single mRNA. Using this unique tool, we found that varying stoichiometry of G, M, T protein expression resulted in significant differences in iCM reprogramming efficiency and quality. Moreover, we found the optimal stoichiometry for iCM reprogramming to be a relative high level of Mef2c protein expression and low levels of Gata4 and Tbx5 expression. By addition of an antibiotic selection cassette to the optimal G, M, T combination (MGT), we further enriched population of transduced fibroblasts yielding an even higher efficiency of generation of functional iCMs. Our approach provides a valuable platform for further mechanistic studies of direct cardiac reprogramming.

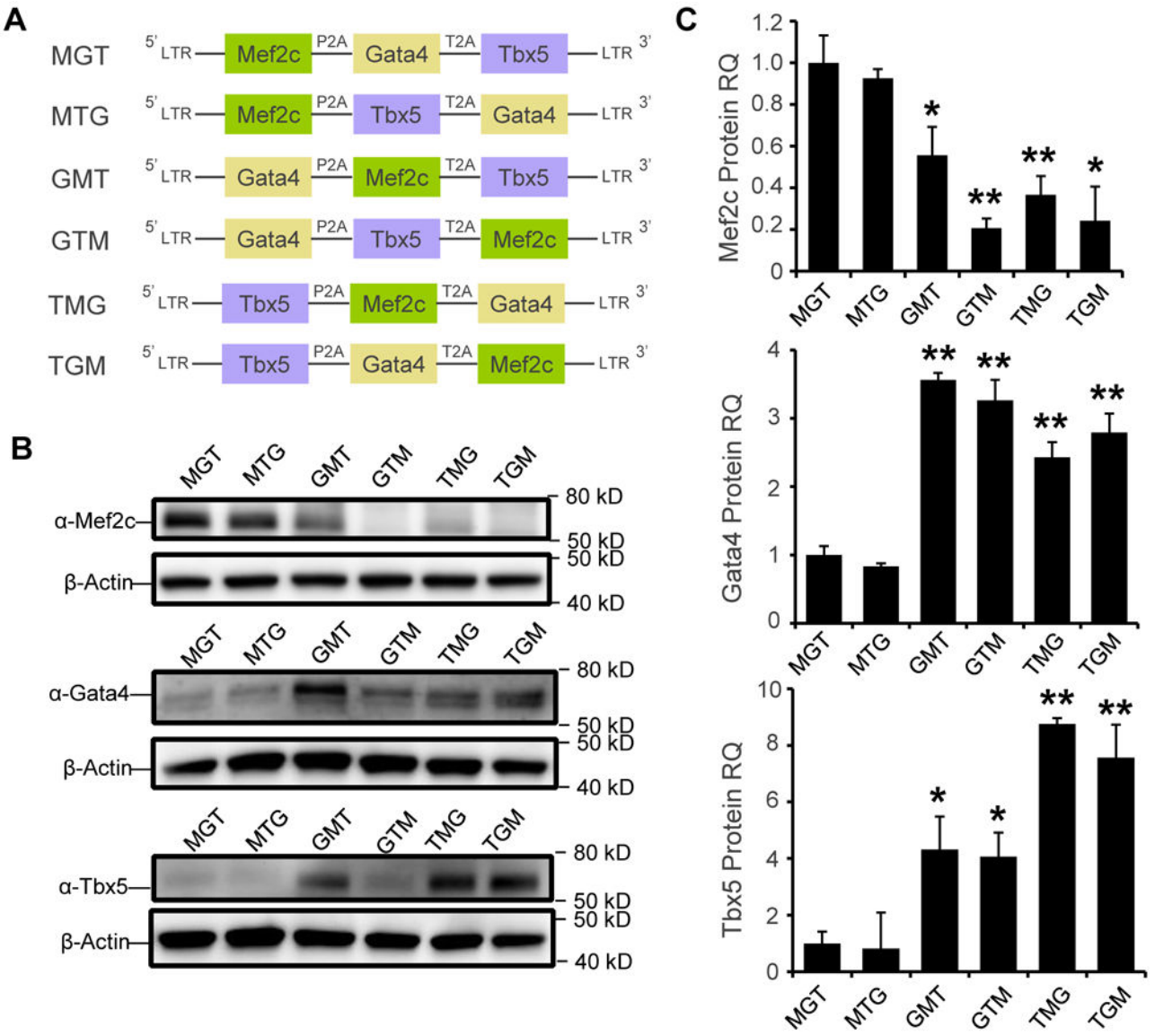

Figure 1. A complete set of polycistronic vectors that result in different G, M, T protein levels

(A) Diagram of the six polycistronic vectors with G, M, T in different splicing orders. Reprogramming factors were cloned in all possible orders (separated by identical P2A and T2A sequences) into the retroviral vector pMX. (B) Western blot analysis of CFs expressing each of the 6 different polycistronic vectors. Cell lysate was collected at 3 days post infection. (C) Quantification of G, M, T protein expression levels. Error bars represent standard error of mean. *, $p<0.05$; ** $p<0.01$.

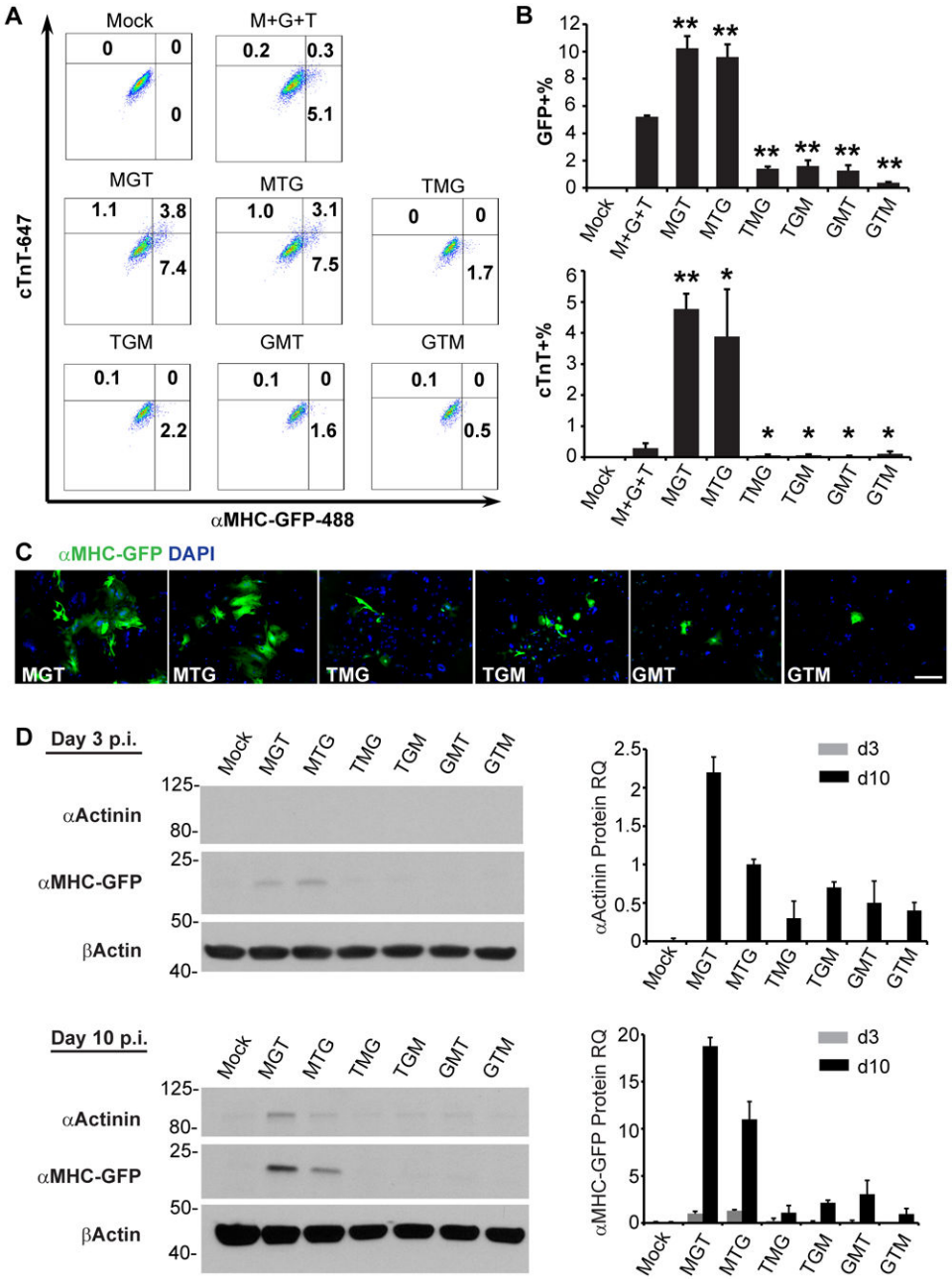

Figure 2. iCM reprogramming efficiencies using the 6 polycistronic vectors

(A) Representative FACS plots of cTnT+, αMHC-GFP+ and double positive iCMs. (B) Quantification of the FACS data. (C) Representative ICC images of iCMs generated by using each of the 6 constructs. Note that only 2 vectors resulted in higher reprogramming efficiencies relative to others. Scale bar: 200μm. *, $p<0.05$; ** $p<0.01$. (D) Western blot analysis of GFP and α-actinin expression in the CFs expressing each of the 6 constructs. Quantification of GFP and α-actinin expression from three independent experiments is shown in the bar graph. Error bars represent standard error of mean.

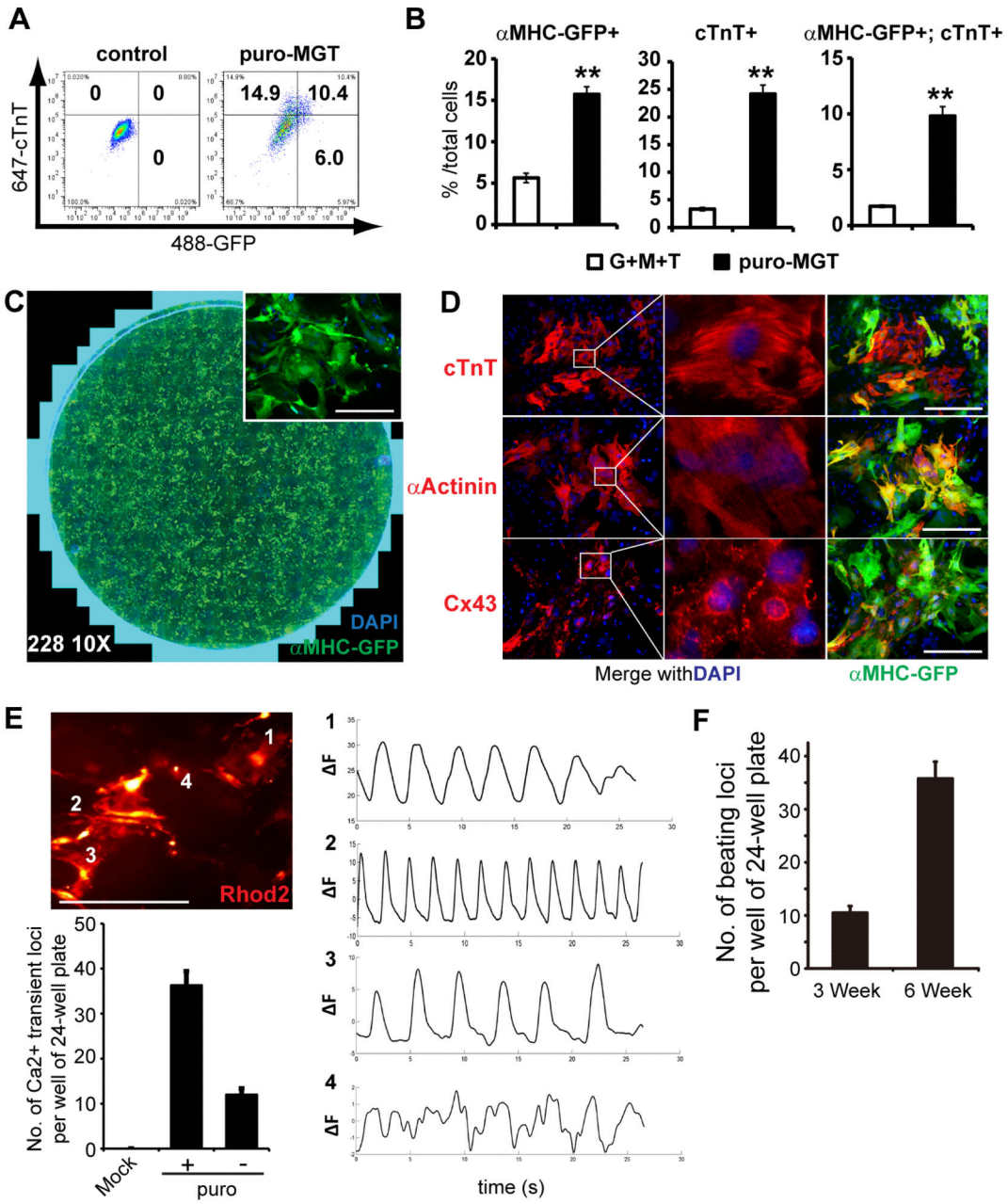

Figure 3. Further enhancement of iCM reprogramming efficiency using puro-MGT

(A) Representative FACS plots of cTnT+, αMHC-GFP+ and double positive iCMs. (B) Quantification of FACS data. ** p<0.01. (C) Stitched (from 228 individual image) ICC picture showing iCMs (GFP positive) in one well of 24-well plate under optimized condition. Inset showing a representative high resolution image of an iCM cluster. (D) ICC of cardiac markers cTnT, α-actinin and Cx43 double labeling with αMHC-GFP. The middle panels are enlarged areas from the left-hand panels as indicated by the white rectangles. Note the striated pattern of sarcomeres in the top two middle panels and Cx43 positive spots at the cell-cell contacts in the bottom middle panel. Scale bar: 200μm. (E) iCM calcium transients measured by Rhod3 dye labeling. Each trace corresponds to the spot numbered in

the upper-left panel. Scale bar: 200μm. Quantification is shown in the lower left histogram. (F) Quantification of the number of beating iCM loci over time. Error bars represent standard error of mean.

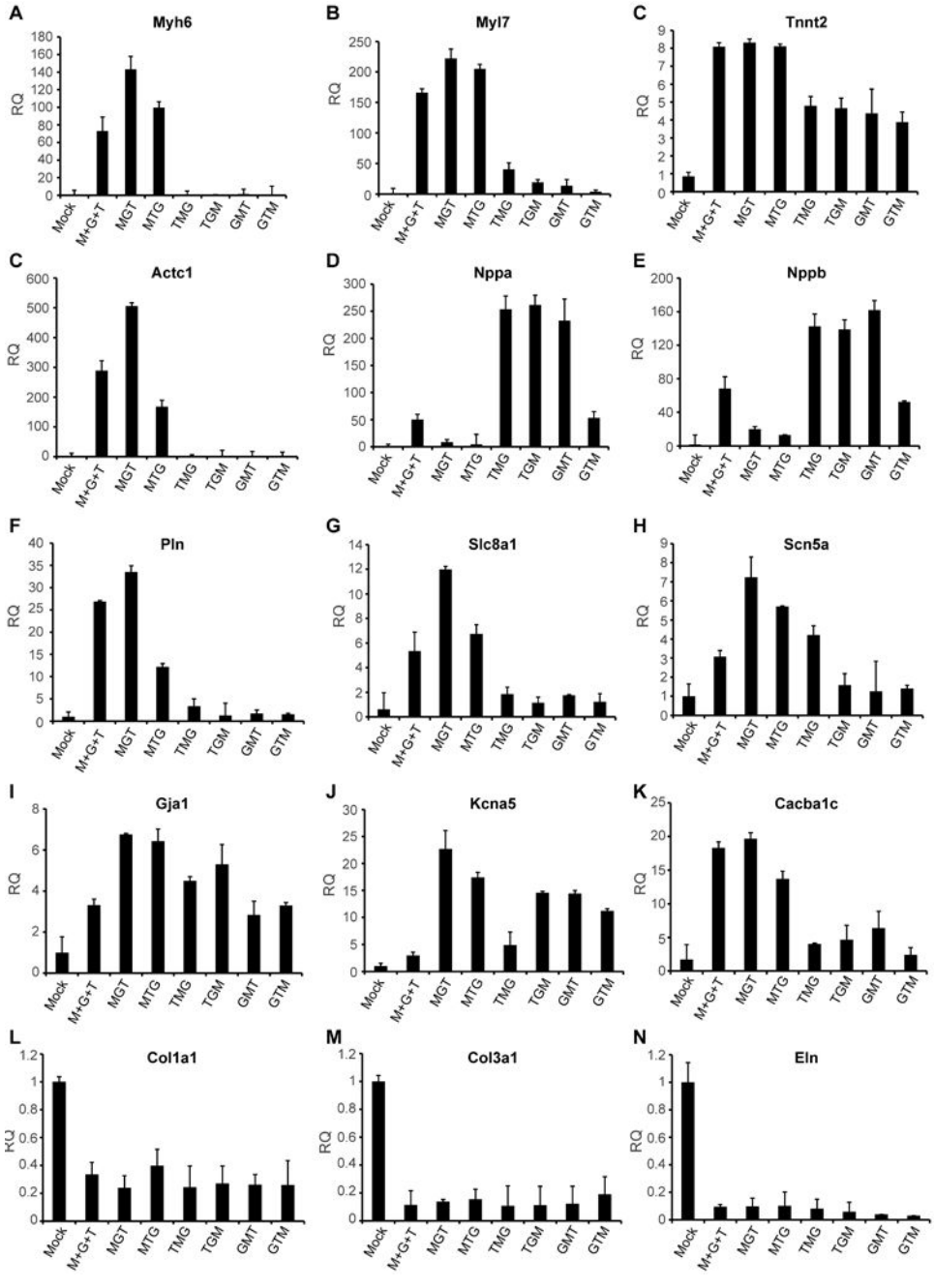

Figure 4. Gene expression analyses of CFs expressing each individual polycistronic vector

Relative expression of a panel of CM or fibroblast marker genes in CFs infected with each of the 6 polycistronic vectors compared with CFs infected with mock (dsRed) or pooled G +M+T at 10 days post infections. Error bars represent standard error of mean.